

A water-soluble [60]fullerene-derivative stimulates chlorophyll accumulation and has no toxic effect on *Chlamydomonas reinhardtii**

Jakub Lang¹, Mariia Melnykova¹, Michele Catania¹, Alicja Ingot¹, Aleksandra Zyss¹, Katarzyna Mikruta¹, Daria Firgolska¹, Agata Wieremiejczuk¹, Izabela Książek¹, Maciej Serda²✉, Paweł Nalepa², Bartosz Pluciński¹, Aleksandra Giza³ and Paweł Jedynak¹✉

¹Department of Plant Physiology and Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University in Krakow, Kraków, Poland; ²Institute of Chemistry, Faculty of Mathematics, Physics and Chemistry, University of Silesia in Katowice, Katowice, Poland; ³Department of Plant Biotechnology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University in Krakow, Kraków, Poland

Chlamydomonas reinhardtii (WT 2137) P. A. Dang. (*Volvocales*, *Chlorophyceae*) is a green microalgae serving as a suitable model in scientific research and a promising industrial biotechnology platform for production of biofuel, hydrogen and recombinant proteins. Fullerenes (C₆₀) are allotropic carbon nanoparticles discovered in 1985 and used in biomedical studies since the early 1990s, when water solubilization methodologies were developed. Recently, surface-modified hydroxylated derivatives of fullerenes were proven to enhance algal growth and drought tolerance in plants. Here, a novel type of water-soluble [60]fullerene derivative with 12 glycine residues (GF) has been synthesized and tested for acute toxicity (up to 50 µg/ml) and as a potential biostimulant of algal growth. The effects of GF on pigment composition and growth rate of *Chlamydomonas reinhardtii* were systematically investigated. Our results suggest that GF was not toxic, and no negative change in the pigment content and no stress symptoms were observed. No changes in the photosynthetic parameters based on the fluorescence of chlorophyll *a* in Photosystem II (NPQ, F_v/F_m, F_v/F_o, PI and RC/ABS) were observed. The GF had no effect on cell size and growth rate. At a concentration of 20 µg/ml, GF stimulated chlorophyll accumulation in 3-day-old cultures.

Key words: *Chlamydomonas reinhardtii*, algae, [60]fullerene-derivative, chlorophyll, toxicity

Received: 31 March, 2019; revised: 16 May, 2019; accepted: 01 June, 2019; available on-line: 07 July, 2019

✉e-mail: p.a.jedynak@gmail.com, pawel.jedynak@uj.edu.pl (PJ); maciej.serda@us.edu.pl (MS)

*A preliminary report on this subject was presented at the 46th Winter School of the Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University in Krakow, Zakopane, Poland, February 11th–15th, 2019

Acknowledgements: The authors thank the Department of Plant Physiology and Biochemistry FBB&B for the support. Maciej Serda thanks the National Science Center (Poland) for the support (UMO-2016/23/D/NZ7/00912).

Abbreviations: GF, water-soluble [60]fullerene derivative with 12 glycine residues; F_v/F_m, maximal quantum efficiency of Photosystem II; F_v/F_o, oxygen-evolving complex efficiency; PI, performance index; RC/ABS, the force generated by the RC concentration per antenna chlorophyll

sion agriculture. Nanoparticles (Rizvi *et al.*, 2017), and the fullerenes among them, arouse potential interests for biotechnology. Pristine fullerenes are water-insoluble carbon spheres, typically containing about 60 atoms of carbon and fullerene C₆₀ has been the best studied one so far. Fullerenes became quickly recognized as generally detrimental or toxic to microbes, cyanobacteria, algae, plants and animals (Lin *et al.*, 2009; Landa *et al.*, 2012; Chen *et al.*, 2018; 2019). Thus, their usage as biostimulants is limited, but on the other hand they can act as potential cytotoxic agents (Lucafo *et al.*, 2013; Franskevych *et al.*, 2017), and as carriers for targeted drug-delivery in cancer therapies (Prylutska *et al.*, 2015; Lapin *et al.*, 2017). In addition, fullerene supplementation may enhance the uptake and accumulation of toxic substances in plants grown on polluted soil (De La Torre-Roche *et al.*, 2012).

In nanomedical literature, it is well described that the surface of fullerenes can be chemically modified, mainly by using the Bingel-Hirsch and Prato reactions, altering fullerene physical and chemical properties (reviewed in detail in: Goodarzi *et al.*, 2017). Such a change affects the fullerenes biological activities and modulates their application as photosensitizers in photodynamic therapies and as *in vivo* transfection agents (Maeda-Mamiya *et al.*, 2010; Sharma *et al.*, 2011). The effects of water-soluble derivatives of fullerenes containing multiple hydroxyl (fullerol or fullerenol), amine and carboxyl groups were extensively studied in plants and animals (Ma & Liang *et al.*, 2010). Fullerenol may penetrate through the cell membrane (Foley *et al.*, 2002). Carbon nanomaterials smaller than 500 nm in length can easily get through the plant cell wall. Fullerenes may passively pass across cell membranes (due to their high affinity to the hydrophobic phase) (Bedrov *et al.*, 2008), but diffusion of fullereneol is a few orders of magnitude lower (Qiao *et al.*, 2007). However, fullerene derivatives can be absorbed by endocytosis in animal (Zhang *et al.*, 2009) and possibly plant cells, as endocytosis of carbon nanotubes was evidenced in the plant cells (Liu *et al.*, 2009). Both, the fullerenes and fullereneol were shown to accumulate in the cytoplasm of living tobacco (Kole *et al.*, 2013; Husen & Siddigi, 2014) and rice cells (Lin *et al.*, 2009).

Fullerene and its derivatives can act both, as prooxidative (Sayes *et al.*, 2004; Grebowski *et al.*, 2013; Huang *et al.*, 2014; Yin *et al.*, 2015) and antioxidative agents (Prylutska *et al.*, 2008; Injac *et al.*, 2013; Sachkova *et al.*, 2017; Roy *et al.*, 2018; Tyurin *et al.*, 2018). It was found that

INTRODUCTION

Engineered carbon nanomaterials have become more and more prevalent in industry, medicine and in preci-

fullerenol had generally no effect or tended to stimulate growth of photosynthetic organisms. In *Arabidopsis thaliana*, the hypocotyl length was increased and no other effects were observed (Gao *et al.*, 2011). Fullerol treatment of *Momordica charantia* resulted in biomass increase by 54%, an increase in yield by 128% and a significantly enhanced accumulation of phytomedicines (Kole *et al.*, 2013). Fullerol binds water molecules, thus greatly improving resistance of the sugar beet (*Beta vulgaris*) to drought stress (Borišev *et al.*, 2016). In addition, fullerene application had decreased oxidative stress elicited by water-deficient conditions (Borišev *et al.*, 2016). Little is known about its impact on algae. Fullerol treatment had increased cell density of *Pseudokirchneriella subcapitata* algae cultures (Gao *et al.*, 2011). However, the negative effects of surface-modified fullerenes were also evidenced. The $C_{70}(C(COOH)_2)_{4-8}$ derivative had caused auxin transport abnormalities and deformation of the root tip, as well as had decreased the shoot growth in *Arabidopsis thaliana* (Liu *et al.*, 2010). Fullerol had also severely damaged the root cells of *Allium cepa* (Chen *et al.*, 2010). However, the use of nanoparticles, including carbon allotropes, raises a question about their safety in the food chain and their environmental safety (Rico *et al.*, 2011; Wang *et al.*, 2018). Presence of C_{60} in the sewage had a negative effect on activated sludge and methanogenesis during anaerobic digestion (Zhao *et al.*, 2018), but removal of fullerenols was efficient (>90%) and had no significant effect on the microorganism's activity (Wang *et al.*, 2011). Thus, the presence of fullerene poses little risk of pollution in terms of wastewater production. Taking the above into consideration, it can be assumed that at low cost and low risk of serious environmental pollution, fullerenes may be potentially used as stimulants of growth of microorganisms cultured in bioreactors.

Chlamydomonas reinhardtii is a green microalgae serving as a model in scientific research and a promising industrial biotechnology platform for production of biofuel, hydrogen and recombinant proteins (Scranton *et al.*, 2015; Rasala *et al.*, 2015; Scoma *et al.*, 2015). Here, a novel type of water-soluble [60]fullerene derivative containing 12 glycine residues (GF) (Fig. 1) has been synthesized and tested for acute toxicity, and as a potential regulator for improvement of algal growth. Particularly,

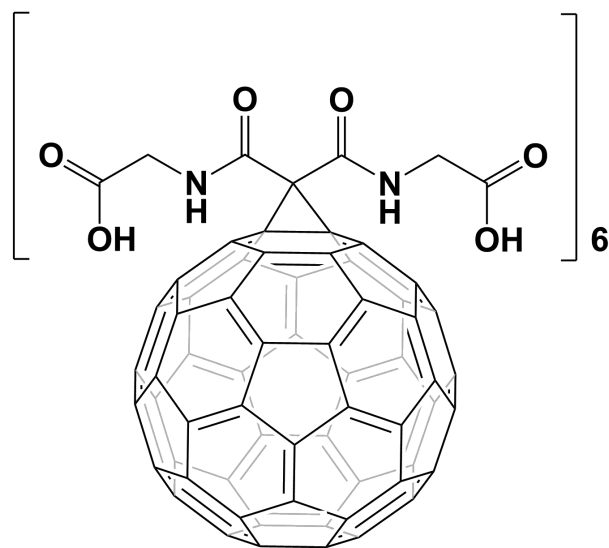


Figure 1. Chemical structure of GF, a [60]fullerene derivative containing 12 glycine residues

the effect of GF on pigment composition and growth rate of *C. reinhardtii* were systematically investigated. Our results suggest that GF was not toxic and could moderately stimulate pigment accumulation in a *C. reinhardtii* culture.

MATERIALS AND METHODS

Materials for synthesis of fullerene derivatives. All compounds used were of reagent grade or better, solvents were used as received unless otherwise specified. The following reagents were used as received: C_{60} (99.5+%, MER Corp.), glycine (Acros Organics), DBU (1,8-diaza-bicyclo (5. 4. 0) undec-7-ene, Sigma Aldrich), malonic acid (Sigma Aldrich), CBr_4 (Sigma Aldrich), and sodium hydride (Acros Organics). Nuclear magnetic resonance spectra were measured on a Bruker Avance III 500 MHz NMR Spectrometer with tetramethylsilane as an internal standard. MS spectra for water-insoluble compounds were collected using an Autoflex II MALDI-TOF (Matrix Assisted Laser Desorption and Ionisation- Time Of Flight) mass spectrometer, and for water-soluble [60]fullerene derivatives by an MS electrospray ionization time-of-flight (ESI-microTOF) mass spectrometer, both instruments from Bruker Daltonics Inc. High resolution spectra were performed using Shimadzu IT (Ion Trap) and TOFLC-MS System, and flash chromatography was performed using Isolera Flash Purification System. The purity of all compounds was assessed using an Agilent1260 equipped with a DAD detector at 260 nm, RP-column: Eclipse plus C18 (3.5 μ m); flow rate 0.5 ml/min.

Highly water-soluble glycine derivative of [60]fullerene was synthesized using previously developed methodology (Serda *et al.*, 2018a; Serda *et al.*, 2018b).

Biological material and growth conditions. Axenic cultures of *Chlamydomonas reinhardtii* (WT 2137) P. A. Dang. (*Volvocales*, *Chlorophyceae*) were obtained from Dr. Itzhak Ohad, Hebrew University, Department of Biological Chemistry, Givat Ram, Jerusalem, Israel, in the 1990s and cultured in our laboratory (Prasad *et al.*, 1998). Cultures were grown under continuous white light (80 μ mol \times m $^{-2}$ \times s $^{-1}$ OSRAM L36W/77, Germany) with shaking (125 rpm) in a Sager-Granick medium (Sager-Granick *et al.*, 1953), supplemented with 100 mM mannitol as an osmoprotectant, and sodium acetate (75 mM), and citrate (1.7 mM) as sources of organic carbon, with addition of soluble, surface-modified (containing amino acid residues) fullerenes to final concentration of 0; 20; 40 and 50 μ g/ml. Samples were collected after 3, 6 and 9 days of cultivation.

Cell counting. The cells were counted using LUNA-Fl Dual fluorescence cell counter (Logos Biosystems, South Korea).

Fluorescence of chlorophyll *a* in Photosystem II. Photosynthetic parameters were measured using HANDY PEA fluorimeter equipped with Liquid-Phase Chlorophyll Fluorescence Adapter for Handy PEA (Hansatech Instruments, United Kingdom). All fluorescence measurements were conducted after 15 minutes of adaptation of algal samples to darkness. Then, the maximal quantum efficiency of Photosystem II (F_v/F_m), oxygen-evolving complex efficiency (F_v/F_o), the force generated by the chlorophyll in reaction center concentration per antenna chlorophyll (RC/ABS) and performance index (PI) were simultaneously measured. Additionally, the non-photochemical quenching (NPQ) was measured us-

ing Open FluorCam FC 800-O (Photon Systems Instruments, spol. s r. o., Czech Republic).

Pigment composition. Chlorophyll and carotenoid content were estimated spectrophotometrically (UV-Vis spectrophotometer, JASCO, United States), according to Lichtenthaler (1987).

Statistical analysis. For group comparison, we used the Kruskal-Wallis rank sum test (Hollander & Douglas, 1973). Multiple comparisons between concentrations after Kruskal-Wallis test were done by the *kruskalmc* function from the *pgirmess* package (Siegel & Castellan, 1988), using the R (version 3.2.4) system for statistical computing (R Core Team, 2019). *P*-values less than 0.05 were considered to be significant. Graphs were produced using Origin 7.0 (OriginLab). Each experiment was repeated in pentaplicate.

RESULTS

The GF had no statistically significant effect on accumulation of carotenoids and chlorophyll *b* (Fig. 2B and D). The chlorophyll *a* content was significantly ($P < 0.05$) increased in 3-day-old cultures treated with 20 $\mu\text{g/ml}$ of GF (Fig. 2A) and the same was observed for total (*a* + *b*) chlorophyll concentration (Fig. 2C). Any other combination of GF concentration and time had no visible effect on the chlorophyll (*a*, *b* or total) content. No statistically significant effect on chlorophyll *a/b* ratio (Fig. 2E), nor chlorophyll to carotenoid ratio (Fig. 2F) was observed. The number of cells (Fig. 3A) in the control and GF-treated samples was similar, and the cell size decreased during the growth period in both,

the control and tested cultures (Fig. 3B). Measurement of the chlorophyll *a* fluorescence in Photosystem II is a widely used technique of estimation of the physiological state of plants, for details see the following reviews (Maxwell & Johnson, 2000; Misra *et al.*, 2012; Kalaji *et al.*, 2014). We have measured selected parameters related to chlorophyll *a* fluorescence in Photosystem II. That allowed estimation of Photosystem II (F_v/F_m) efficiency, the number of chlorophyll molecules per reaction center (RC/ABS) that in turn allows the estimation of the number of antennas in one reaction center, and F_v/F_0 related to the efficiency of oxygen-evolving complex and the Performance Index (PI), which allow estimation of the overall photosynthesis efficiency. GF had no significant effect on any of these parameters (Fig. 3C–F). We have also measured fluorometrically the non-photochemical quenching (NPQ, the efficiency of thermal dissipation of energy from excited chlorophyll molecules during stressful, high-light conditions) of 9-day-old cultures and no differences between the GF-treated and the control samples were observed (Fig. 4).

DISCUSSION

Our results indicate that at a concentration up to 50 $\mu\text{g ml}^{-1}$, GF had no toxic effect on *C. reinhardtii*. GF had no negative effect on the pigment accumulation and did not interfere with photosynthesis and cell growth. No stress symptoms were observed. Pigment content and the chlorophyll to carotenoid ratio change dramatically under suboptimal conditions (e. g. salt stress) (Sairam & Tyagi, 2004; Hussein *et al.*, 2014) and may be accompa-

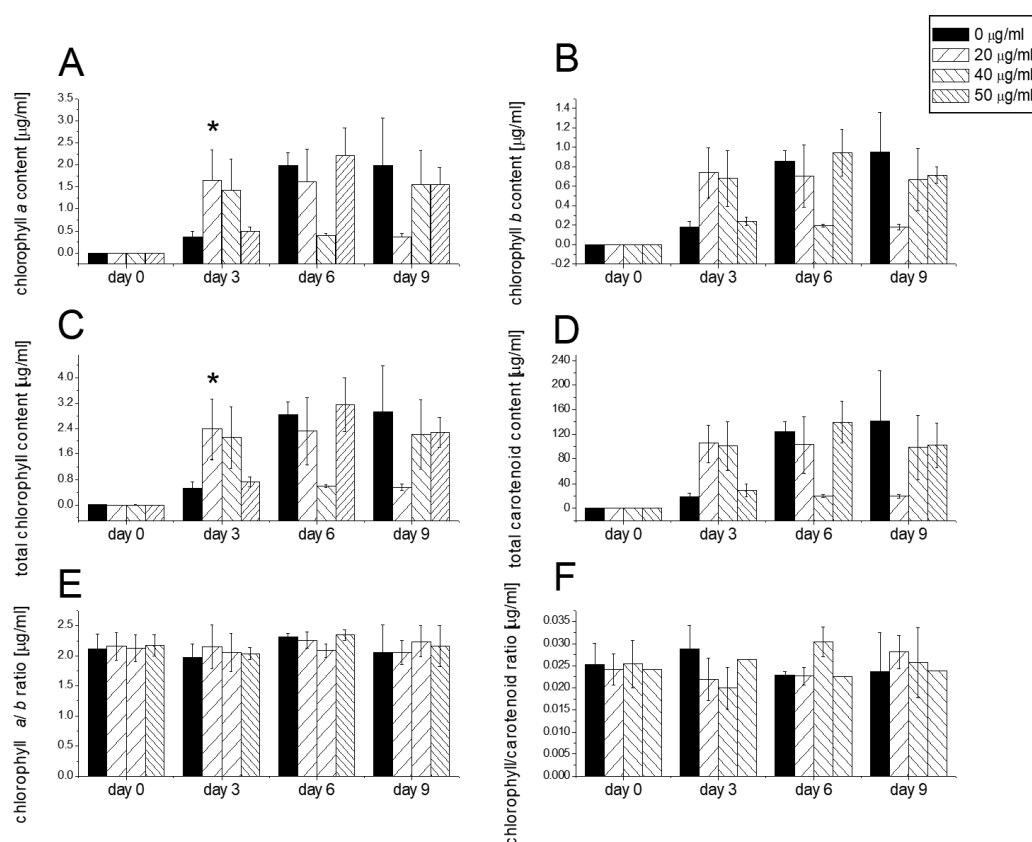


Figure 2. The effect of GF on pigment composition of *Chlamydomonas reinhardtii*. Changes in the content of chlorophyll *a* (A) and *b* (B), as well as total chlorophyll (C), and total carotenoid (D); (E) the chlorophyll *a/b* ratio; (F) the chlorophyll to carotenoid ratio. Statistically significant differences ($P < 0.05$) are indicated with *. Error bars – S.D.

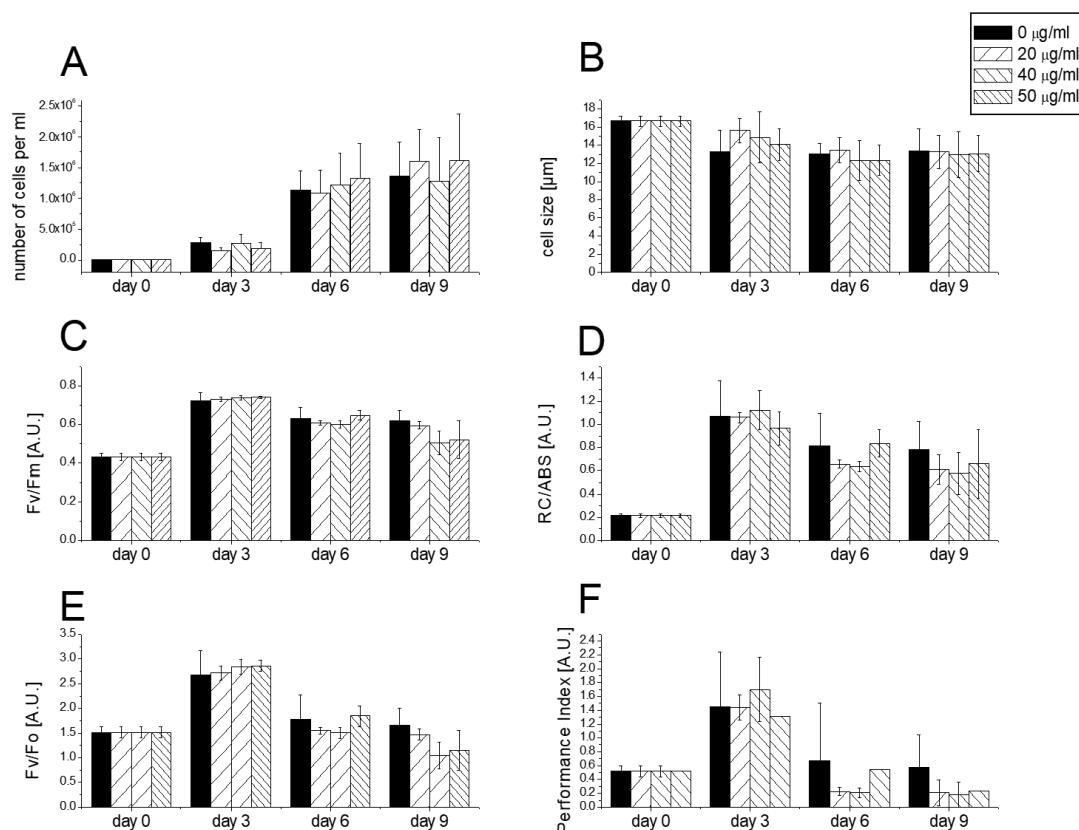


Figure 3. The effect of GF on cell size, number of cells per ml and selected photosynthetic parameters.

The number of counted cells (A) and cell size (B) measured by a cell counter. The results of fluorimetric measurements of the efficiency of photosystem II – F_v/F_m (C), the number of chlorophyll molecules per reaction center (D), the efficiency of water-evolving complex (E) and Performance Index (F) indicating overall photosynthesis efficiency. All data were checked for statistically significant differences ($P < 0.05$). Error bars – S.D.

nied by a significant increase of NPQ (Pak *et al.*, 2009; Dongsansuk, 2013), suggesting weaker protection from higher intensities of light. No such change was observed during our experiment. On the contrary, we observed transient stimulation of chlorophyll *a* accumulation that might be beneficial. No other stimulating effect was observed at the tested concentrations, thus at the current

stage of research there is no evidence that GF may be considered as a biostimulant of algal growth. However, its low toxicity and little interference with physiology of *C. reinhardtii* suggest that it can be developed as an excellent delivery system for entrapped ions and growth regulators (as fullerenes may act as cages with time-delayed release of molecules) or as a tool for DNA delivery. Since the GF surface is negatively charged, it may bind cationic molecules. It was shown that such properties may be used for reducing toxicity of heavy metals (Anderson & Barron, 2005), which may be complexed using hydroxyfullerenes. Furthermore, GF-based technology will be further developed and focused on the role of GF under stress conditions (as surface modified fullerenes enhance particular stress tolerance in plants), lipid accumulation and production of important biomolecules.

Conflicts of interest

The authors declare that there is no conflict of interest regarding publication of this article.

LITERATURE

- Anderson R, Barron AR (2005) Reaction of hydroxyfullerene with metal salts: a route to remediation and immobilization. *J Am Chem Soc* 127: 10458–10459. <https://doi.org/10.1021/ja051659d>
- Bedrov D, Smith GD, Davande H, Li L (2008) Passive transport of C_{60} fullerenes through a lipid membrane: a molecular dynamics simulation study. *J Phys Chem B* 112: 2078–2084. <https://doi.org/10.1021/jp075149c>
- Borišev M, Borišev I, Župunski M, Arsenov D, Pajević S, Čurčić Ž, Vasin J, Djordjevic A (2016) Drought impact is alleviated in sugar

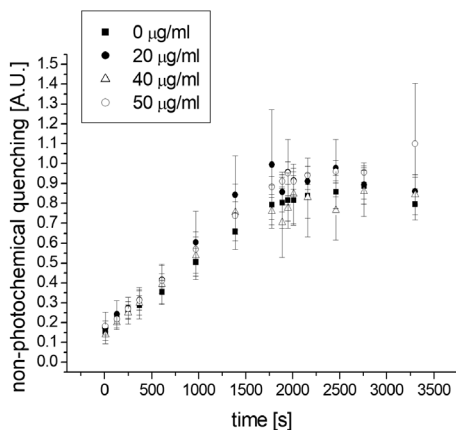


Figure 4. The effect of GF on non-photochemical quenching in *C. reinhardtii*

The fluorimetric measurements of NPQ in 9-day old cultures of *C. reinhardtii*. Dark-adapted cultures were irradiated with actinic (saturating photosynthesis) light per 1700 s, then the fluorescence was recorded under pseudo-dark conditions. Statistically significant differences ($P < 0.05$) are indicated with *. Error bars – S.D.

- beets (*Beta vulgaris* L.) by foliar application of fullereneol nanoparticles. *PLoS One* **11**: e0166248. <https://doi.org/10.1371/journal.pone.0166248>
- Chen M, Sun Y, Liang J, Zeng G, Li Z, Tang L, Zhu Y, Jiang D, Song B (2019) Understanding the influence of carbon nanomaterials on microbial communities. *Environ Int* **126**: 690–698. <https://doi.org/10.1016/j.envint.2019.02.005>
- Chen M, Zhou S, Zhu Y, Sun Y, Zeng G, Yang C, Xu P, Yan M, Liu Z, Zhang W (2018) Toxicity of carbon nanomaterials to plants, animals and microbes: Recent progress from 2015–present. *Chemosphere* **206**: 255–264. <https://doi.org/10.1016/j.chemosphere.2018.05.020>
- Chen R, Ratnikova TA, Stone MB, Lin S, Lard M, Huang G, Hudson JS, Ke PC (2010) Differential uptake of carbon nanoparticles by plant and mammalian cells. *Small* **6**: 612–617. <https://doi.org/10.1002/sml.200901911>
- De La Torre-Roche R, Hawthorne J, Deng Y, Xing B, Cai W, Newman LA, Wang C, Ma X, White JC (2012) Fullerene-enhanced accumulation of p,p'-DDE in agricultural crop species. *Environ Sci Technol* **46**: 9315–9323. <https://doi.org/10.1021/es301982w>
- Dongsansuk A (2013) The performance of PSII efficiency and growth response to salt stress in three rice varieties differing in salt tolerance. In *Molecular Stress Physiology of Plants*. Gyana Ranjan Rout Ananth Bandhu Das, eds, pp. 87–131 Springer Dordrecht Heidelberg New York London
- Foley S, Crowley C, Smaih M, Bonfils C, Erlanger BF, Seta P, Larroque C (2002) Cellular localization of a water-soluble fullerene derivative. *Biochem Biophys Res Commun* **294**: 116–125. [https://doi.org/10.1016/S0006-291X\(02\)00445-X](https://doi.org/10.1016/S0006-291X(02)00445-X)
- Franskevych D, Palyvoda K, Petukhov D, Prylutska S, Grynyuk I, Schuetze K, Drobot L, Matyshevska O, Ritter U (2017) Fullerene C₆₀ penetration into leukemic cells and its photoinduced cytotoxic effects. *Nanoscale Res Lett* **12**: 40. <https://doi.org/10.1186/s11671-016-1819-5>
- Gao J, Wang Y, Folta KM, Krishna V, Bai W, Indeglia P, Georgieva A, Nakamura H, Koopman B, Moudgil B (2011) Polyhydroxy fullerenes (fullerols or fullerolins): beneficial effects on growth and lifespan in diverse biological models. *PLoS One* **6**: e19976. <https://doi.org/10.1371/journal.pone.0019976>
- Goodarzi S, Da Ros T, Conde J, Sefat F, Mozafari M (2017) Fullerene: biomedical engineers get to revisit an old friend. *Materials Today* **20**: 460–480. <https://doi.org/10.1016/j.mat.2017.03.017>
- Grebowski J, Kazmierska P, Krokosz A (2013) Fullereneols as a new therapeutic approach in nanomedicine. *Biomed Res Int* **2013**: 751–913. <https://doi.org/10.1155/2013/751913>
- Hollander M, Wolfe DA (1973) *Nonparametric Statistical Methods*. New York: John Wiley & Sons. pp. 115–120.
- Huang YY, Sharma SK, Yin R, Agrawal T, Chiang LY, Hamblin MR (2014) Functionalized fullerenes in photodynamic therapy. *J Biomed Nanotechnol* **10**: 1918–1936. <https://doi.org/10.1166/jbn.2014.1963>
- Husen A, Siddiqi KS (2014) Carbon d fullerene nanomaterials in plant system. *J Nanobiotechnology* **12**: 16. <https://doi.org/10.1186/1477-3155-12-16>
- Hussein MM, Mehanna H, Zaki SN, Nagwan FAH (2014) Influences of salt stress and foliar fertilizers on growth, chlorophyll and carotenoids of jojoba plants. *Middle East J Agric Res* **3**: 221–226.
- Injac R, Prijatelj M, Strukelj B (2013) Fullereneol nanoparticles: toxicity and antioxidant activity. *Methods Mol Biol* **1028**: 75–100. https://doi.org/10.1007/978-1-62703-475-3_5
- Kalaji HM, Schansker G, Ladle RJ, Goltsev V, Bosa K, Allakhverdiev SI, Brestic M, Bussotti F, Calatayud, Dąbrowski P, Elsheery NI, Ferroni L, Guidi L, Hogewoning SW, Jajoo A, Misra AN, Nebauer SG, Pancaldi S, Penella C, Poli D, Pollastri M, Romanowska-Duda ZB, Rutkowska B, Seródio J, Suresh K, Szulc W, Tambussi E, Yannicari M, Zivcak M (2014) Frequently asked questions about *in vivo* chlorophyll fluorescence: practical issues. *Photosynth Res Nov* **122**: 121–158. <https://doi.org/10.1007/s11120-014-0024-6>
- Kole C, Kole P, Randunu KM, Choudhary P, Podila R, Ke PC, Rao AM, Marcus RK (2013) Nanobiotechnology can boost crop production and quality: first evidence from increased plant biomass, fruit yield and phytomedicine content in bitter melon (*Momordica charantia*). *BMC Biotechnol* **13**: 37. <https://doi.org/10.1186/1472-6750-13-37>
- Landa P, Vankova R, Androva J, Hodek J, Marsik P, Storchova H, White JC, Vanek T (2012) Nanoparticle-specific changes in *Arabidopsis thaliana* gene expression after exposure to ZnO, TiO₂, and fullerene soot. *J Hazard Mater* **241**: 242–255. <https://doi.org/10.1016/j.jhazmat.2012.08.059>
- Lapin NA, Krzykawska-Serda M, Dilliard S, Mackeyev Y, Serda M, Wilson LJ, Curley SA, Corr SJ (2017) The effects of non-invasive radiofrequency electric field hyperthermia on biotransport and bio-distribution of fluorescent [60]fullerene derivative in a murine orthotopic model of breast adenocarcinoma. *J Controlled Release* **260**: 92–99. <https://doi.org/10.1016/j.jconrel.2017.05.022>
- Lichtenthaler HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic membranes. *Methods Enzymol* **148**: 350–382
- Lin S, Reppert J, Hu Q, Hudson JS, Reid ML, Ratnikova TA, Rao AM, Luo H, Ke PC (2009) Uptake, translocation, and transmission of carbon nanomaterials in rice plants. *Small* **5**: 1128–1132. <https://doi.org/10.1002/sml.200801556>
- Liu Q, Chen B, Wang Q, Shi X, Xiao Z, Lin J, Fang X (2009) Carbon nanotubes as molecular transporters for walled plant cells. *Nano Lett* **9**: 1007–1010. <https://doi.org/10.1021/nl803083u>
- Liu Q, Zhao Y, Wan Y, Zheng J, Zhang X, Wang C, Fang X, Lin J (2010) Study of the inhibitory effect of water-soluble fullerenes on plant growth at the cellular level. *ACS Nano* **4**: 5743–5748. <https://doi.org/10.1021/nn101430g>
- Lucafo M, Gerdol M, Pallavicini A, Pacor S, Zorzet S, Da Ros T, Prato M, Sava G (2013) Profiling the molecular mechanism of fullerene cytotoxicity on tumor cells by RNA-seq. *Toxicology* **314**: 183–192. <https://doi.org/10.1016/j.tox.2013.10.001>
- Ma H, Liang XJ (2010) Fullerenes as unique nanopharmaceuticals for disease treatment. *Sci China Chem* **53**: 2233–2240. <https://doi.org/10.1007/s11426-010-4118-5>
- Maeda-Mamiya R, Noiri E, Isobe H, Nakanishi W, Okamoto K, Doi K, Sugaya T, Izumi T, Homma T, Nakamura E (2010) *In vivo* gene delivery by cationic tetraamino fullerene. *Proc Natl Acad Sci USA* **107**: 5339–5344. <https://doi.org/10.1073/pnas.0909223107>
- Maxwell K, Johnson GN (2000) Chlorophyll fluorescence – a practical guide. *J Exp Bot* **51**: 659–668. <https://doi.org/10.1093/jexbot/51.345.659>
- Misra AN, Misra M, Singh R (2012) Chlorophyll fluorescence in plant biology. In *Biophysics*, Misra AN ed, pp 171–192. Rijeka, Croatia: In-Tech. 10.5772/1877
- Pak VA, Nabipour M, Meskarbashe M (2009) Effect of salt stress on chlorophyll content, fluorescence, Na⁺ and K⁺ ions content in rape plants (*Brassica napus* L.). *Asian J Agric Res* **3**: 28–37. <https://doi.org/10.3923/ajar.2009.28.37>
- Prasad MN, Drej K, Skawirnska A, Strzalka K (1998) Toxicity of cadmium and copper in *Chlamydomonas reinhardtii* wild-type (WT 2137) and cell wall deficient mutant strain (CW 15). *Bull Environ Contam Toxicol* **60**: 306–311
- Prylutska SV, Grynyuk II (2008) Anti-oxidant properties of C₆₀ fullerenes *in vitro*. *Fuller Nanotub Carbon Nanostruct* **16**: 698–705. <https://doi.org/10.1080/15363830802317148>
- Prylutska SV, Skivka LM (2015) Complex of C₆₀ fullerene with doxorubicin as a promising agent in antitumor therapy. *Nanoscale Res Lett* **10**: 499. <https://doi.org/10.1186/s11671-015-1206-7>
- R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>
- Qiao R, Roberts AP, Mount AS, Klaine SJ, Ke PC (2007) Translocation of C₆₀ and its derivatives across a lipid bilayer. *Nano Lett* **7**: 614–619. <https://doi.org/10.1021/nl062515f>
- Rasala BA, Mayfield SP (2015) Photosynthetic biomanufacturing in green algae: production of recombinant proteins for industrial, nutritional, and medical uses. *Photosynth Res* **123**: 227–239. <https://doi.org/10.1007/s11120-014-9994-7>
- Rico CM, Majumdar S, Duarte-Gardea M, Peralta-Videa JR, Gardea-Torresdey J (2011) Interaction of nanoparticles with edible plants and their possible implications in the food chain. *J Agric Food Chem* **59**: 3485–3498. <https://doi.org/10.1021/jf104517j>
- Rizvi SAA, Saleh AM (2017) Applications of nanoparticle systems in drug delivery technology. *Saudi Pharm J* **26**: 64–70. <https://doi.org/10.1016/j.jsps.2017.10.012>
- Roy P, Bag S, Chakraborty D, Dasgupta S (2018) Exploring the inhibitory and antioxidant effects of fullerene and fullereneol on ribonuclease A. *ACS Omega* **3**: 12270–12283. <https://doi.org/10.1021/acsomega.8b01584>
- Sachkova AS, Kovel ES, Churilov GN, Guseynov OA, Bondar AA, Dubinina IA, Kudryasheva NS (2017) On mechanism of antioxidant effect of fullereneols. *BB Reports* **9**: 1–8. <https://doi.org/10.1016/j.bbrep.2016.10.011>
- Sager R, Granick S (1953) Nutritional studies with *Chlamydomonas reinhardtii*. *Ann New York Acad Sci* **56**: 831–838
- Sairam RK, Tyagi A (2004) Physiological and molecular biology of salinity stress tolerance in plants. *Curr Sci* **86**: 407–420. <https://doi.org/10.1007/1-4020-4225-6>
- Sayes CM, Fortner JD, Guo W, Lyon D, Boyd AM, Ausman KD, Tao YJ, Sitharaman B, Wilson LJ, Hughes JB, West JL, Colvin WL (2004) The differential cytotoxicity of water-soluble fullerenes. *Nano Letters* **4**: 1881–1887. <https://doi.org/10.1021/nl0489586>
- Scoma A, Faraloni C, Giannelli L (2015) Advances in the biotechnology of hydrogen production with the microalga *Chlamydomonas reinhardtii*. *Crit Rev Biotechnol* **35**: 485–496. <https://doi.org/10.3109/07388551.2014.900734>
- Scranton MA, Ostrand JT, Fields FJ, Mayfield SP (2015) *Chlamydomonas* as a model for biofuels and bio-products production. *Plant J* **82**: 523–531. <https://doi.org/10.1111/tpj.12780>
- Serda M, Nalepa P, Musiol R (2018b) Diglicinederivative of [60]fullerene and the methods of synthesis thereof. *Polish Patent Application*, P. 428469.

- Serda M, Ware MJ, Newton JM, Sachdeva S, Krzykawska-Serda M, Nguyen L, Law J, Anderson AO, Curley SA, Wilson LJ, Corr SJ (2018a) *Nanomedicine (Lond)* **13**: 2981–2993.
- Sharma SK, Chiang IY, Hamblin MR (2011) Photodynamic therapy with fullerenes *in vivo*: reality or a dream? *Nanomedicine* **6**: 1813–1825. <https://doi.org/10.2217/nnm.11.144>.
- Siegel S, Castellan J (1988) Non parametric statistics for the behavioural sciences, pp 213–221. MacGraw Hill Int., New York.
- Tyurin DP, Kolmogorov FS, Cherepkova IA, Charykov N, Semenov K, Keskinov V, Safyannikov NA, Pukharenyu V, Letenko DG, Sege-da TA, Shaimardanov Z (2018) Antioxidant properties of fullerene-d. *Nanosystems: Physics, Chemistry, Mathematics* **9**: 798–810. <https://doi.org/10.17586/2220-8054-2018-9-6-798-810>.
- Wang C, Chang XL, Shi Q, Zhang X (2018) Uptake and transfer of (13)C-fullerenols from *Scenedesmus obliquus* to *Daphnia magna* in an aquatic environment. *Environ Sci Technol* **29**: (Epub ahead print) <https://doi.org/10.1021/acs.est.8b03121>.
- Wang Y, Westerhoff P, Hristovski KD (2011) Fate and biological effects of silver, titanium dioxide, and C₆₀ (fullerene) nanomaterials during simulated wastewater treatment processes. *J Hazard Mater* **201–202**: 16–22. <https://doi.org/10.1016/j.jhazmat.2011.10.086>.
- Yin R, Wang M, Huang YY, Landi G, Vecchio D, Chiang IY, Hamblin MR (2015) Antimicrobial photodynamic inactivation with decacationic functionalized fullerenes: oxygen-independent photokilling in presence of azide and new mechanistic insights. *Free Radic Biol Med* **79**: 14–27. <https://doi.org/10.1016/j.freeradbiomed.2014.10.514>.
- Zhang LW, Yang J, Barron AR, Monteiro-Riviere NA (2009) Endocytic mechanisms and toxicity of a functionalized fullerene in human cells. *Toxicol Lett* **191**: 149–157. <https://doi.org/10.1016/j.toxlet.2009.08.017>.
- Zhao L, Ji Y, Sun P, Li R, Xiang F, Wang H, Ruiz-Martinez J, Yang Y (2018) Effects of individual and complex ciprofloxacin, fullerene C[60], and ZnO nanoparticles on sludge digestion: Methane production, metabolism, and microbial community. *Bioresour Technol* **267**: 46–53. <https://doi.org/10.1016/j.biortech.2018.07.024>.